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(54) Title: PROTEASOME REGULATION OF NF-κB ACTIVITY

(57) Abstract

Disclosed herein is a method for regulating the activity of NF-κB in an animal comprising contacting cells of the animal with certain proteasome inhibitors.

Proteasome Regulation of NF-κB Activity

Background of the Invention

1. Field of the Invention

The present invention relates to a method for reducing the cellular content and activity of NF-κB by use of inhibitors of proteasome function or ubiquitin conjugation.

2. Description of Related Art

The transcription factor NF-κB and other members of the rel family of protein complexes play a central role in the regulation of a remarkably diverse set of genes involved in the immune and inflammatory responses (Grilli *et al.*, *International Review of Cytology 143*:1-62 (1993)). For example, NF-κB is required for the expression of a number of immune response genes, including the Ig-κ light chain immunoglobulin gene, the IL-2 receptor α chain gene, the T cell receptor β chain gene, and class I and II major histocompatibility genes. In addition, NF-κB has been shown to be required for a number of genes involved in the inflammatory response, such as the TNF-α gene and the cell adhesion genes, E-selectin, I-cam, and V-cam. NF-κB is also required for the expression of a large number of cytokine genes such as IL-2, IL-6, G-CSF, and IFN-β. Finally, NF-κB is essential for the expression of the human immunodeficiency virus (HIV).

In the cytosol, there is a soluble proteolytic pathway that requires ATP and involves covalent conjugation of the cellular proteins with the small polypeptide ubiquitin ("Ub") (Hershko *et al.*, *A. Rev. Biochem. 61*:761-807 (1992); Rechsteiner *et al.*, *A. Rev. Cell. Biol. 3*:1-30 (1987)). Thereafter, the conjugated proteins are hydrolyzed by a 26S proteolytic complex containing a 20S degradative particle called the proteasome (Goldberg, *Eur. J. Biochem. 203*:9-23 (1992); Goldberg *et al.*, *Nature 357*:375-379 (1992)). This multicomponent system is known to catalyze the selective degradation of

and appears to be part of the 26S proteasome complex, which rapidly degrades proteins conjugated to ubiquitin. This protease, referred to as multipain, has been identified in muscle and plays an essential role in the ATP-ubiquitin-dependent pathway.

The complex formed between multipain and proteasome *in vitro* appears very similar or identical to the 1500 kDa Ub-conjugate, degrading enzyme, or 26S proteolytic complex, isolated from reticulocytes and muscle. The complexes contain the characteristic 20-30 kDa proteasome subunits, plus a number of larger subunits, including the six large polypeptides found in multipain. The complex formed contains at least 10-12 polypeptides of 40-150 kDa.

A 40 kDa polypeptide regulator of the proteasome, which inhibits the proteasome's proteolytic activities has been purified from reticulocytes and shown to be an ATP-binding protein whose release appears to activate proteolysis. The isolated regulator exists as a 250 kDa multimer and is quite labile (at 42°C). It can be stabilized by the addition of ATP or a nonhydrolyzable ATP analog, although the purified regulator does not require ATP to inhibit proteasome function and lacks ATPase activity. The regulator has been shown to correspond to an essential component of the 1500 kDa proteolytic complex. The regulator appears identical to CF-2 by many criteria. These findings suggest that the regulator plays a role in the ATP-dependent mechanism of the 26S proteasome complex.

There is also a system in the cytosol that generates antigenic particles from endogenously synthesized cellular and viral proteins (Moore *et al.*, *Cell 54*:777-785 (1988); Morrison *et al.*, *J. Exp. Med. 163*:903-921 (1986); Powis *et al.*, *Nature 354*:529-531 (1991); Spies *et al.*, *Nature 351*:323-324 (1991); Townsend *et al.*, *Cell 42*:457-467 (1985); Townsend *et al.*, *Nature 324*:575-577 (1986); Monaco *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 79*:3001-3005 (1982); Monaco, *Immun. Today 13*:173-179 (1992); Yewdell *et al.*, *Adv. Immun. 52*:1-123 (1992); Townsend *et al.*, *J. Exp. Med. 168*:1211-1224 (1988)). Indirect evidence suggests a role for proteolytic particles closely

Biophys. 218:1 (1989); Rivett *et al., J. Biol. Chem. 264*:12,215-12,219 (1989); Tanaka *et al., New Biol. 4*:1-11 (1992)). These include known inhibitors of chymotrypsin-like and trypsin-like proteases, as well as inhibitors of thiol (or cysteine) and serine proteases. In addition, some endogenous inhibitors of proteasome activities have been isolated. These include the 240 kDa and the 200 kDa inhibitors isolated from human erythrocytes (Murakami *et al., Proc. Natl. Acad. Sci. U.S.A. 83*:7588-7592 (1986); Li *et al., Biochemistry 30*:9709-9715 (1991)) and purified CF-2 (Goldberg, *Eur. J. Biochem. 203*:9-23 (1992)). In addition to antibiotic inhibitors originally isolated from actinomycetes (Aoyagi *et al., Proteases and Biological Control*, Cold Spring Harbor Laboratory Press, pp. 429-454 (1975)), a variety of peptide aldehydes have been synthesized, such as the inhibitors of chymotrypsin-like proteases described by Siman *et al.* (WO 91/13904).

Novel molecules can also be obtained and tested for inhibitory activity. As illustrated by the above cited references, various strategies are known in the art for obtaining the inhibitors for a given protease. Compound or extract libraries can be screened for inhibitors using peptidase assays. Alternatively, peptide and peptidomimetic molecules can be designed based on knowledge of the substrates of the protease. For example, substrate analogs can be synthesized containing a reactive group likely to interact with the catalytic site of the protease (see, e.g., Siman *et al.*, WO 91/13904; Powers *et al.*, in *Proteinase Inhibitors*, Barrett *et al.* (eds.), Elsevier, pp. 55-152 (1986)). The inhibitors can be stable analogs of catalytic transition states (transition state analog inhibitors), such as Z-Gly-Gly-Leu-H, which inhibits the chymotrypsin-like activity of the proteasome (Orlowski, *Biochemistry 29*:10289-10297 (1990); see also Kennedy and Schultz, *Biochemistry 18*:349 (1979)).

Various natural and chemical protease inhibitors reported in the literature, or molecules similar to them, include peptides containing an α-diketone or an α-keto ester, peptide chloromethyl ketones, isocoumarins, peptide sulfonyl fluorides, peptidyl boronates, peptide epoxides, and peptidyl diazomethanes (Angelastro *et al., J. Med Chem. 33*:11-13 (1990); Bey *et al.*,

the several peptide α-keto ester inhibitors tested, Z - Leu - Leu - Phe - COOEt was the most potent inhibitor of the chymotrypsin-like activity with a K_i of 53 μM. Many such compounds exist.

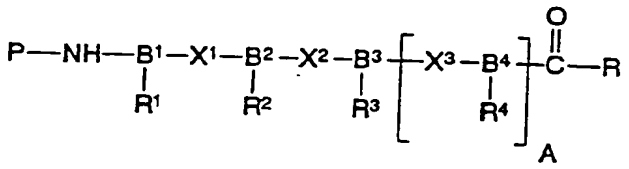
Other tripeptides that have been described in the literature include Ac-Leu-Leu-Leu-H, Ac-Leu-Leu-Met-OR, Ac-Leu-Leu-Nle-OR, Ac-Leu-Leu-Leu-OR, Ac-Leu-Leu-Arg-H, Z-Leu-Leu-Leu-H, Z-Arg-Leu-Phe-H, and Z-Arg-Ile-Phe-H, where OR, along with the carbonyl of the preceding amino acid residue, represents an ester group.

Goldberg, in U.S. Patent Application Serial No. 07/699,184, filed May 13, 1991, discloses that the ATP-ubiquitin-dependent process has been shown to be responsible for the excessive protein degradation that occurs in conditions or disease states in which there is severe loss of body mass and negative nitrogen balance. A method of inhibiting the accelerated or enhanced proteolysis, a method of identifying inhibitors of the process, multipain and proteasome inhibitors are also disclosed.

Goldberg *et al.*, in U.S. Patent Application Serial No. 08/016,066, filed February 10, 1993, disclose methods and drugs that inhibit the processing of antigens for presentation by major histocompatibility complex class I molecules. Specifically, inhibitors of the ATP-ubiquitin-dependent proteolytic pathway are described, which can inhibit MHC-I antigen presentation. These methods and drugs may be useful for the treatment of autoimmune diseases and for reducing rejection of organs and graft transplants. See, also, Michalek *et al., Nature 363*:552-554 (1993).

Tsubuki *et al., Biochem. and Biophys. Res. Commun. 196(3)*:1195-1201 (1993) reported that a tripeptide aldehyde protease inhibitor, benzyloxycarbonyl(Z)-Leu-Leu-leucinal, initiates neurite outgrowth in PC12 cells at an optimal concentration of 30 nM. The following synthetic peptides are also mentioned: Z-Leu-Leu-Gly-H, Z-Leu-Leu-Ala-H, Z-Leu-Leu-Ile-H, Z-Leu-Leu-Val-H, Z-Leu-Leu-Nva-H, Z-Leu-Leu-Phe-H, Z-Leu-Leu-Leu-H, Bz-Leu-Leu-Leu-H, Ac-Leu-Leu-Leu-H, Z-Leu-Leu-Leu.sc, Z-Leu-Leu-

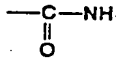
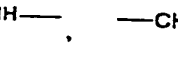
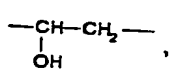
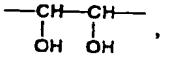
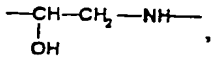
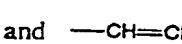
More particularly, the present invention is directed to a method for reducing the cellular content and activity of NF-κB in an animal comprising contacting cells of the animal with a proteasome function or ubiquitin conjugation inhibitor of the structure (1):



(1)

where

P is an amino-group-protecting moiety;

B^1, B^2, B^3, and B^4 are independently selected from the group consisting of  and  ; X^1, X^2, and X^3 are independently selected from the group consisting of  ,  ,  ,  ,  , and  ;

R is a hydrogen, alkyl, acyl, or carboxyl;

R^1, R^2, R^3, and R^4 are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, and $-CH_2-R^5$,

where R^5 is aryl, aralkyl, alkaryl, cycloalkyl or $-Y-R^6$,

where Y is a chalcogen, and R^6 is alkyl; and

A is 0 or 1.

The "animals" referred to herein are preferably mammals. Both terms are intended to include humans.

Brief Description of the Drawings

Figure 1 shows that proteolytic processing of the p60Tth precursor to p50 *in vitro* requires ATP.

could have important application in various areas of medicine, e.g., inflammation, sepsis, AIDS, and the like.

More specifically, the activity of NF-κB is highly regulated (Grilli *et al., International Review of Cytology 143*: 1-62 (1993); Beg *et al., Genes and Development 7*:2064-2070 (1993)). NF-κB comprises two subunits, p50 and an additional member of the rel gene family, e.g., p65 (also known as Rel A). In most cells, the p50 and p65 are present in an inactive precursor form in the cytoplasm, bound to IκB. In addition, the p50 subunit of NF-κB is generated by the proteolytic processing of a 105 kD precursor protein NF-κB_1 (p105), and this processing is also regulated. The sequence of the N-terminal 50 kD portion of p105 is similar to that of p65 and other members of the rel gene family (the rel homology domain). By contrast, the C-terminal 55 kD of p105 bears a striking resemblance to IκB-α (also known as MAD3). Significantly, unprocessed p105 can associate with p65 and other members of the rel family to form a p65/p105 heterodimer. Processing of p105 results in the production of p50, which can form the transcriptionally active p50/p65 heterodimer. The C-terminal IκB-α-homologous sequence of p105 is rapidly degraded upon processing.

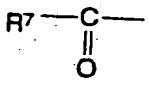
There is another rel-related protein, NF-κB_2 (p100), that is similar to p105 in that it, too, is processed to a DNA binding subunit, p52 (Neri *et al., Cell 67*:1075 (1991); Schmid *et al., Nature 352*:733 (1991); Bours *et al., Molecular and Cellular Biology 12*:685 (1992); Mercurio *et al., DNA Cell Biology 11*:523 (1992)). Many of the structural and regulatory features of p100 are similar to p105. In addition, the p100 protein can also form a heterodimer with p65 and other rel family members.

In summary, the transcriptional activity of heterodimers consisting of p50 and one of the many rel family proteins, such as p65, can be regulated by at least two mechanisms. First, the heterodimers associate with IκB-α to form an inactive ternary cytoplasmic complex. Second, the rel family members associate with p105 and p100 to form inactive complexes. The ternary complex can be activated by the dissociation and destruction of IκB-α, while

processing reaction (e.g., ATP/Mg^{++} dependency) suggested to the present inventors that the ATP-dependent protease complex of the ubiquitin-mediated protein degradation pathway was involved (i.e. proteasome; Rechsteiner, 1991, Goldberg, *Eur. J. Biochem. 203*:9-23 (1992), Hershko *et al., Annu. Rev. Biochem. 61*:761-807 (1992)). However, this structure was only known to catalyze the complete degradation of proteins to small acid-soluble peptides and was not believed capable of processing precursors to generate active proteins, such as p50 NF-κB.

Using a variety of experimental approaches, the present inventors have proven that the proteasome is indeed required for the processing of p105 to p50. First , it was found that the p105/p60*Tth* proteins are not processed in mammalian cell cytoplasmic extracts depleted of proteasome activity. However, addition of purified 26S proteasomes to these depleted extracts restores the processing activity. Second, specific inhibitors of the proteasome block the formation of p50 in mammalian cell extracts and *in vivo*. Third, mammalian p105 is processed to p50 in *Saccharomyces cerevisiae in vivo*, and a mutant in the chymotrypsin-like activity of the proteasome results in a significant decrease in p105 processing. p60*Tth* is ubiquitinated in vitro and this ubiquitination is a pre-requisite for p105 processing.

As mentioned above, the C-terminal half of the p105 (p105C') is rapidly degraded during the formation of p50 and the sequence of p105C' is remarkably similar to that of IκB. Because of the similarity in the structures and activities of p105C' and IκB-α, the present inventors initiated studies to determine whether the proteasome is also involved in the inactivation of IκB-α. IκB-α is rapidly degraded in response to NF-κB inducers and this degradation has been shown to be necessary for the activation (Mellits *et al., Nucleic Acids Research 21(22)*:5059-5066 (1993); Henkel *et al., Nature 365*:182-185 (1993); Beg *et al., Molecular and Cellular Biology 13(6)*:3301-3310 (1993)). The present inventors have now shown that IκB-α degradation and the activation of NF-κB is indeed blocked by inhibitors of proteasome function or ubiquitin conjugation.

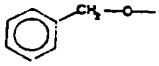


(2)

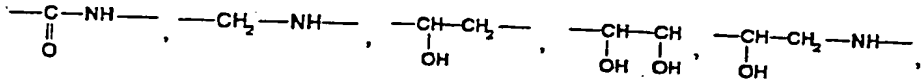
and R^7 is alkyl, aryl, alkaryl, aralkyl, alkoxy, aryloxy, alkaryloxy, or aralkoxy.

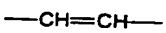
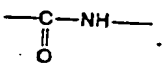
Where R^7 is alkyl, it is preferably alkyl of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof. Additionally, where R^7 is alkaryl, aralkyl, alkoxy, alkaryloxy, or aralkoxy, the alkyl moiety thereof is also preferably one having from 1 to 4 carbon atoms.

Where R^7 is aryl, it is preferably aryl of from 6 to 10 carbon atoms, e.g., phenyl or naphthyl, which may, if desired, be ring substituted. Additionally, where R^7 is alkaryl, aralkyl, aryloxy, alkaryloxy, or aralkoxy, the aryl moiety thereof is also preferably one having from 6 to 10 carbon atoms.

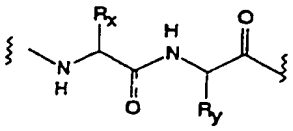
It is more preferred that R^7 be alkyl or aralkoxy, most preferably methyl or benzyloxy, i.e., 

In structure (1), X represents a peptide bond or an isostere that can be used as a peptide bond replacement in the proteasome inhibitors to increase bioavailability and reduce hydrolytic metabolism. As noted above, X can be

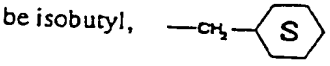
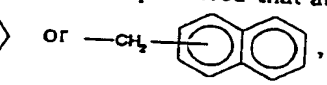


or  . Preferably, X is  .

Introduction of these moieties into the proteasome inhibitors results in the following:

 peptide bond

As noted above, A in structure (1) can be either 0 or 1. Thus, when A is 0, the amino acid residue within the brackets is not present and the inhibitor is a tripeptide. Similarly, where A is 1, the amino acid residue within the brackets *is* present and the inhibitor is a tetrapeptide. It is preferred that A be 0.

It is preferred that R^1 and R^2 in structure (1) be independently selected from the group consisting of alkyl and $—CH_2—R^5$. More preferably, R^1 and R^2 are independently selected from the group consisting of alkyl groups of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof, e.g., isopropyl, isobutyl, sec-butyl, t-butyl, or $—CH_2—R^5$, where R^5 is cycloalkyl or naphthyl. It is more preferred that at least one of R^1 and R^2 be isobutyl,  or  , and most preferred that both R^1 and R^2 be isobutyl.

Where R^3 is alkyl, it is preferably alkyl of from 1 to 4 carbon atoms, e.g., methyl, ethyl, propyl, butyl, or isomers thereof, which groups may be substituted or unsubstituted.

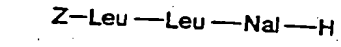
Where R^3 is aryl, it is preferably aryl of from 6 to 10 carbon atoms, e.g., phenyl or naphthyl, which groups may be substituted or unsubstituted.

Where R^3 is a substituted alkyl, it is preferably an alkyl of from 1 to 4 carbon atoms substituted with at least one aryl group of from 6 to 10 carbon atoms or at least one cycloalkyl group, preferably a cycloalkyl group having 5 or 6 carbon atoms, which groups may be substituted or unsubstituted.

Where R^3 is substituted aryl, it is preferably substituted with at least one alkyl group of from 1 to 4 carbon atoms, which groups may be substituted or unsubstituted.

Where R^3 is cycloalkyl, it is preferably cycloalkyl of from 5 to 6 carbon atoms, e.g., cyclopentyl or cyclohexyl, which groups may be substituted or unsubstituted.

Where R^3 is substituted cycloalkyl, it is preferably substituted with at least one aryl group of from 6 to 10 carbon atoms or at least one alkyl group,



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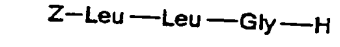

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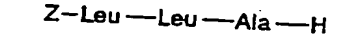

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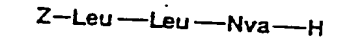

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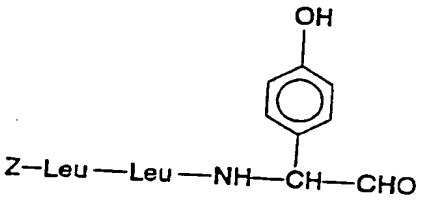

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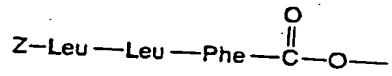

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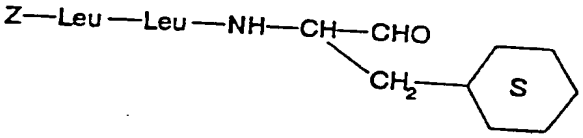

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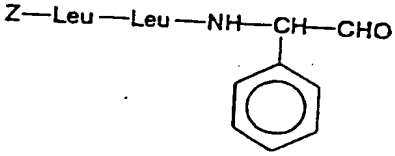
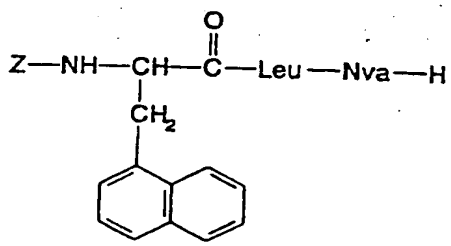


(18)

(19)



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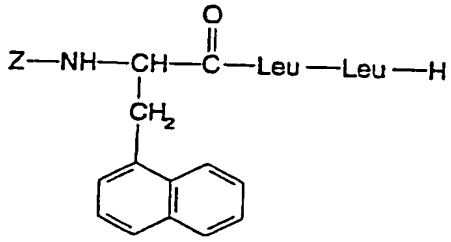

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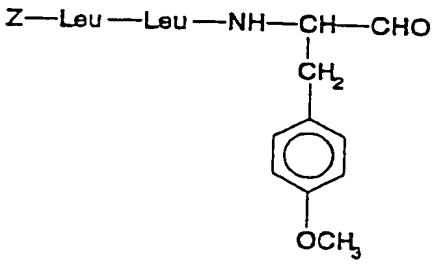

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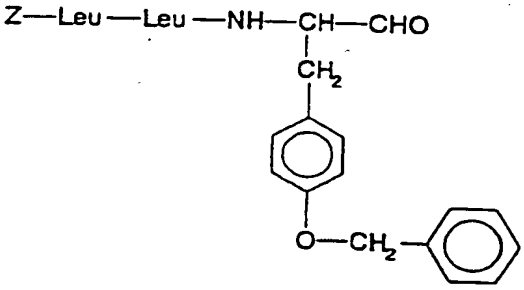

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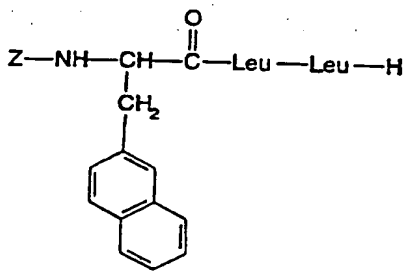

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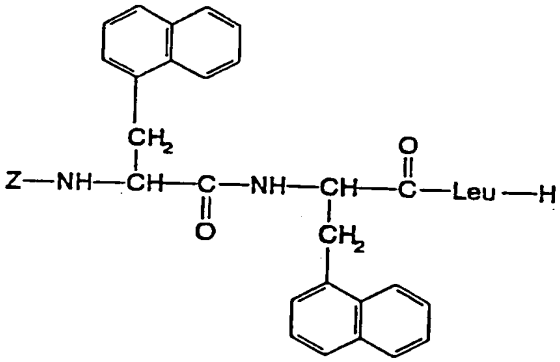
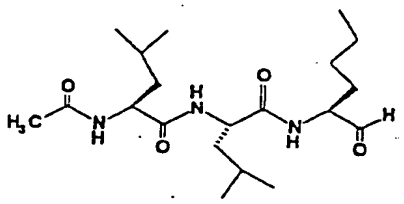


(36)

Most preferably, the proteasome inhibitors are:



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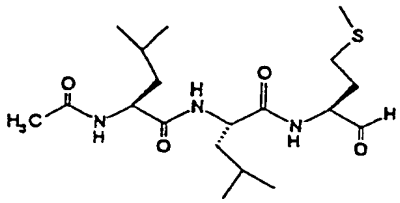

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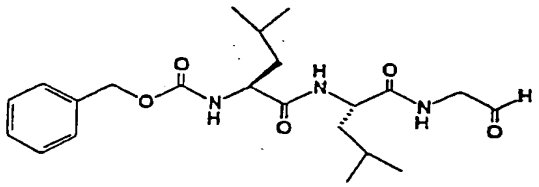

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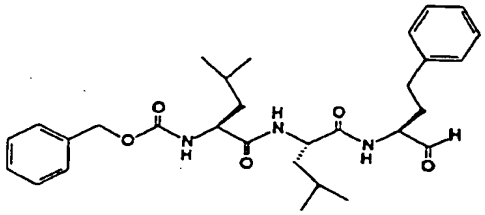

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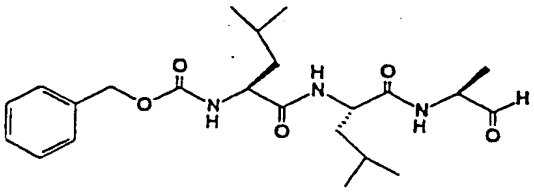

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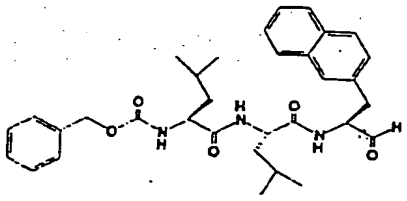

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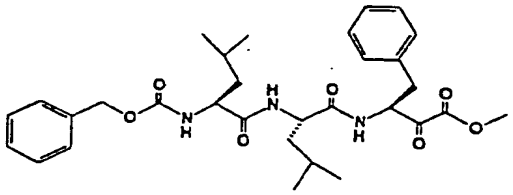

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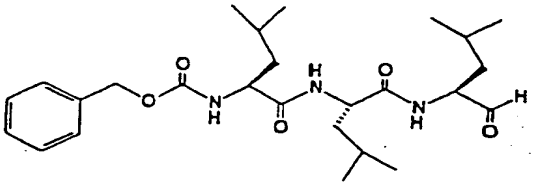

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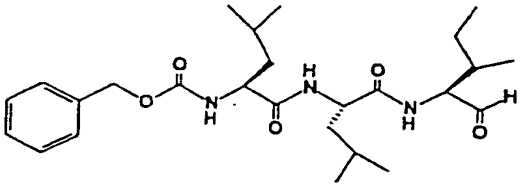

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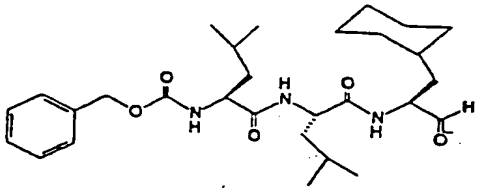

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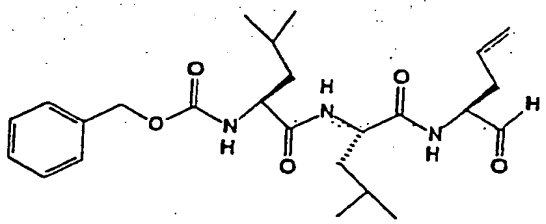

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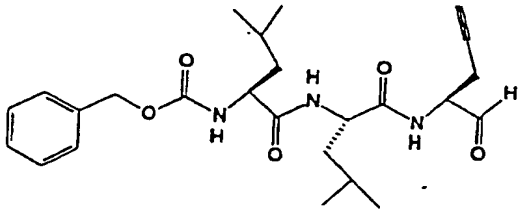

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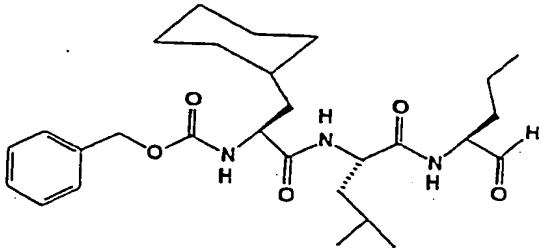

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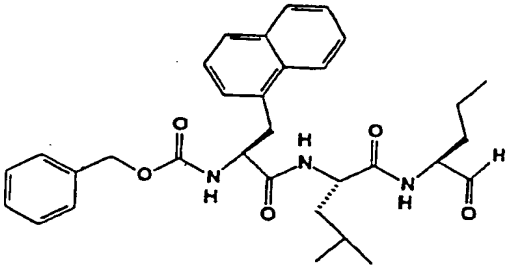

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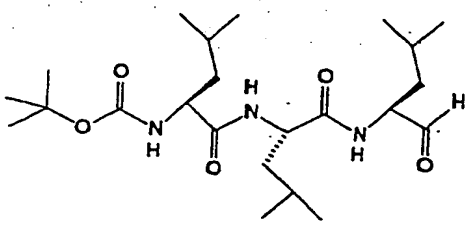

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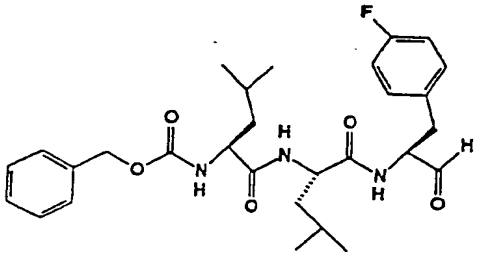

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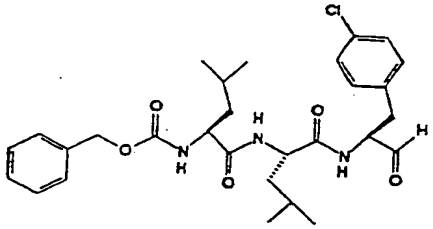

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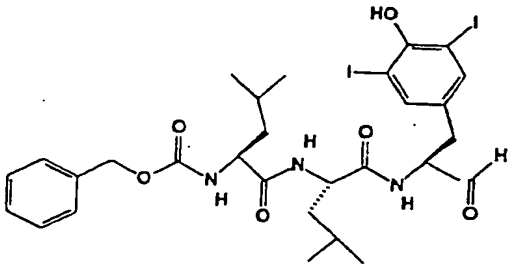

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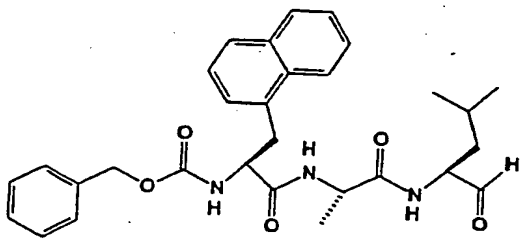
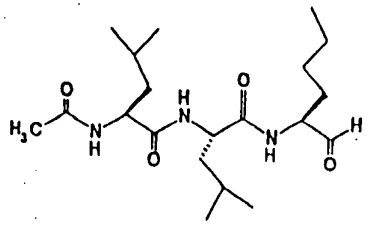
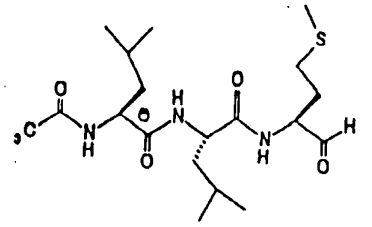


(74)

The present invention relates to a method for reducing the cellular content and activity of NF-κB in an animal comprising contacting cells of the animal with inhibitors of proteasome function or ubiquitin conjugation. In the present method, the accelerated proteolysis is inhibited by interfering with the ATP-Ub-dependent pathway at one or more possible steps (e.g., by interfering with activity of the 26S proteasome complex, or by interfering with activity of one of its components).

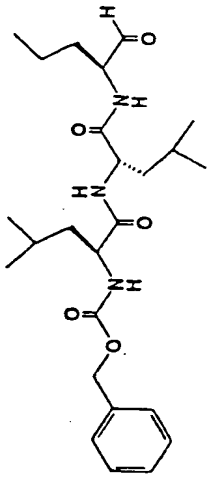
A particularly useful approach to testing drug candidates for their ability to inhibit the ATP-ubiquitin-dependent degradative process is to do so in cultured cells in which a short-lived protein whose degradation is ubiquitin-dependent is produced. Inhibition of the process leads to accumulation of the protein in the cytosol. The extent to which the protein accumulates in the cytosol can be determined, using known methods. For example, a potential inhibitor of the process can be introduced into cultured cells producing a short-lived enzyme and the extent to which the enzyme is present in the cytosol in the presence of the potential inhibitor can be compared with the extent to which it occurs in its absence. Accumulation of the enzyme in the presence of the potential inhibitor is indicative of inhibition of the ATP-ubiquitin-dependent processes by the potential inhibitor being tested. Cultured cells, such as COS cells, which are stably transformed with a gene encoding a short-lived protein whose degradation is ubiquitin-dependent (e.g., a short-lived enzyme, such as a mutant β-galactosidase from *E. coli*, whose half-life is about 15 minutes and whose

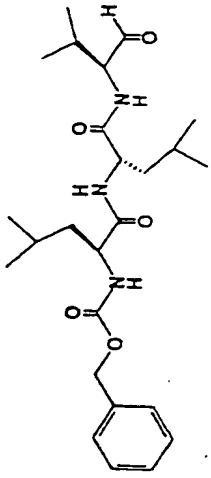
TABLE I: PROTEASE SELECTIVITY OF N-ACETYL TRIPEPTIDE ALDEHYDES

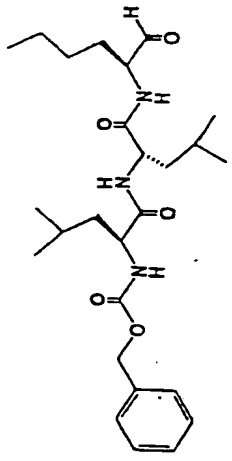
Inhibitor	K_i (nM)			
	20 S[a]	26 S[b]	Cat B[c]	Calpain[d]
MG 101 	140*	1,000	6	5
MG 102 	1,000	28,000	94	120

(a) Rabbit muscle. SDS-activated. Substrate: Suc-LLVY-AMC.
(b) Rabbit muscle. Substrate: Suc-Suc-LLVY-AMC-AMC. [Mg:ATP] = 2 mM.
(c) Bovine spleen. Substrate: Z-RR-AMC. [DTT] = 2 mM, [EDTA] = 5 mM, pH = 5.5, T = 37°C.
(d) Rabbit muscle, 80 kD catalytic subunit. Substrate: Suc-LLVY-AMC. [$CaCl_2$] = 1 mM, [DTT] = 2 mM, pH = 7.8, T = 20°C.

MG 115	21	78	10	10
MG 120	94	560	36	14
MG 114	47	120	7	12
MG 110	48	180	100	62







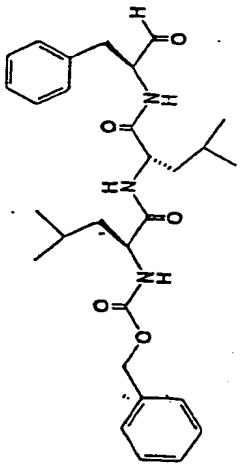
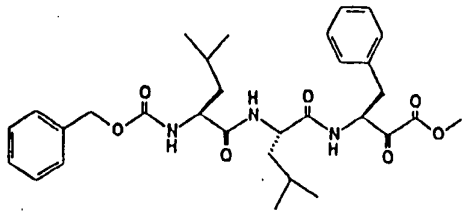


TABLE III: PROTEASE SELECTIVITY OF CARBOXY-ACTIVATED TRIPEPTIDES [a]

Inhibitor	K_i (nM)			
	20 S	26 S	Cat B	Calpain
MG 113 	690	1,300	<4	45

(a) Same reaction conditions as listed in footnotes of Table I.

Data also show that MG 101 is an inhibitor of the 26S ATP-dependent protease and an inhibitor of the proteasome (macropain, multi-catalytic protease) (Table IV).

Table IV

MG 101 Inhibits Different Forms of Muscle Proteasome

Enzyme Preparation	No Inhibitor: Relative Catalytic Efficiency	MG 101: K_i (μM)
20S Proteasome	1	7
26S Proteasome Complex	2	3
20S Proteasome + Muscle 180 kDa Activator	50	0.6
20S Proteasome + SDS	140	0.14

The inhibitors can be used *in vitro* or *in vivo*. They can be administered by any number of known routes, including orally, intravenously, intramuscularly, topically, and by infusion (Platt et al., U.S. Patent No. 4,510, 130; Badalamente *et al., Proc. Natl. Acad. Sci. U.S.A. 86*:5983-5987 (1989); Staubli *et al., Brain Research 444*:153-158 (1988)) and will generally be administered in combination with a physiologically acceptable carrier (e.g., physiological saline). The effective quantity of inhibitor to be given will be determined empirically and will be based on such considerations as the particular inhibitor used, the condition of the individual, and the size and weight of the individual. They can be administered alone or in combination with another inhibitor or an inhibitor of another pathway.

Table V summarizes data for the inhibition of the 20S proteasome by various tripeptide aldehyde inhibitors.

Table V

Protease Selectivity of Miscellaneous Tripeptide Aldehydes

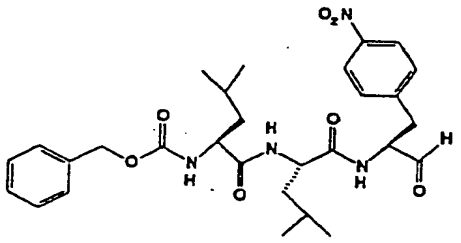
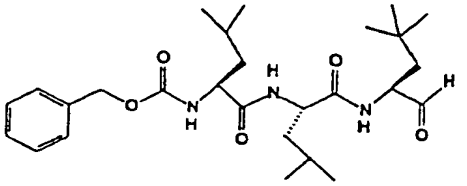
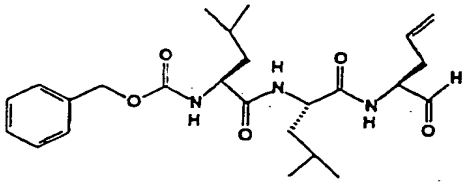
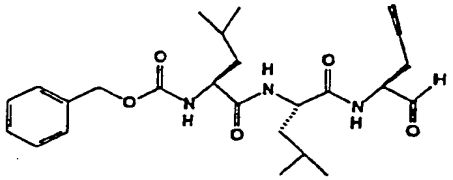
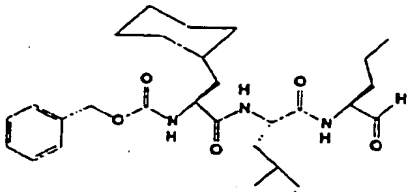
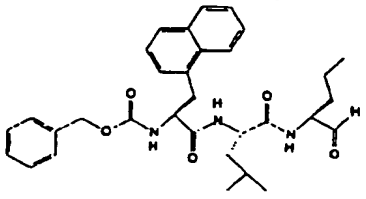
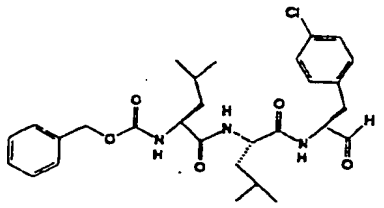
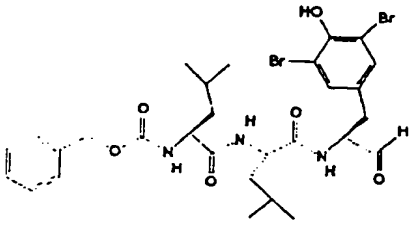
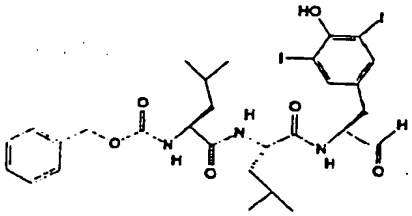
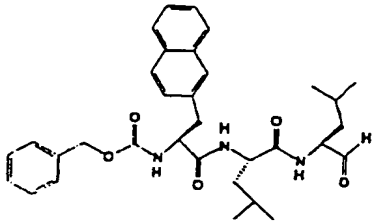
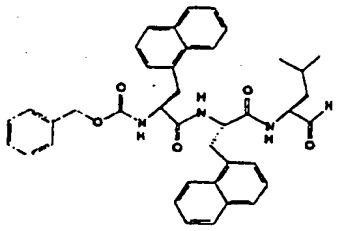
Compound		K_i (nM)
MG 135		290
MG 136		1,000
MG 139		20
MG 140		28
MG 141		50
MG 142		0.3

Table V

Protease Selectivity of Miscellaneous Tripeptide Aldehydes

Compound		K_i (nM)
MG 158		7
MG 160		51
MG 161		64
MG 165		0.24
MG 166		0.035

Example 1

Preparation of Z-L-leucine-L-leucine-L-norvalinal

a) Boc-L-norvaline N,O-dimethylhydroxylamide

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (443 mg) in one portion was added to a mixture of N-Boc-L-norvaline dicyclohexylammonium salt (838 mg), N,O-dimethylhydroxylamine hydrochloride (215 mg), 1-hydroxybenzotriazole monohydrate (340 mg), and N-methylmorpholine (0.28 ml) in dimethyl formamide (DMF, 20 ml) at 0°C. The mixture was stirred at 0°C for 2 hours, then at room temperature for 40 hours. The reaction was quenched with water (80 mL) and the mixture was extracted with ethyl acetate (EtOAc, 3 x 100 mL). The combined organic layers were washed with aqueous 10% hydrogen chloride (HCl), saturated sodium bicarbonate ($NaHCO_3$), and brine, then dried over anhydrous magnesium sulfate ($MgSO_4$), filtered, and evaporated to afford the product (546 mg) as an oil.

b) Z-L-leucine-L-leucine-L-norvaline N,O-dimethylhydroxylamide

A solution of N-Boc-L-norvaline N,O-dimethylhydroxylamide (546 mg) and trifluoroacetic acid (8 mL) in methylene chloride (20 mL) was stirred at 0°C for 3 hours. The solvent was evaporated under reduced pressure and the residue was dried under vacuum. To this flask was added Z-L-leucine-L-leucine (794 mg), 1-hydroxybenzotriazole monohydrate (340 mg), N-methylmorpholine (0.28 mL), and DMF (20 mL). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (442 mg) was then added at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for 24 h. The reaction was quenched with water (40 mL) and the mixture was extracted with EtOAc (3 x 60 mL). The combined organic layers were washed with aqueous 10% HCl, saturated $NaHCO_3$, and brine, then dried over

b) Z-L-leucine-L-leucine-L-leucine N,O-dimethylhydroxylamide

A solution of N-Boc-L-leucine-L-leucine N,O-dimethylhydroxylamide (923 mg) and trifluoroacetic acid (10 mL) in methylene chloride (20 mL) was stirred at 0°C for 3 hours. The solvent was evaporated under reduced pressure and the residue was dried under vacuum. A portion of this product (488 mg) was transferred to another flask and was combined with Z-L-leucine (451 mg), 1-hydroxybenzotriazole monohydrate (276 mg), N-methylmorpholine (0.22 mL), and DMF (15 mL). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (357 mg) was then added at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for 42 h. The reaction was quenched with water (50 mL) and the mixture was extracted with EtOAc (3 x 60 mL). The combined organic layers were washed with aqueous 10% HCl, saturated $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$, filtered, and evaporated to afford the product as a white solid. This was further purified by silica gel chromatography (hexane/acetone 80:20, 70:30) to give the title compound (546 mg) as a white solid.

c) Z-L-leucine-L-leucine-L-leucinal

A solution of Z-L-leucine-L-leucine-L-leucine N,O-dimethylhydroxylamide (546 mg) was dissolved in 15 mL dry tetrahydrofuran (THF) and cooled to 0°C. Lithium aluminum hydride (1 M solution in THF, 4.1 mL) was added and the mixture was stirred at 0°C for 30 minutes. Potassium bisulfate (1.39 g) in 30 mL water was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with aqueous 5% HCl, saturated $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$, filtered, and evaporated to afford the product (446 mg) as a white solid. This was further purified by reverse phase HPLC (water/acetonitrile).

c) **Z-L-(2-naphthyl)-Alanine-L-(1-naphthyl)-Alanine-L-Leucine-N,O-dimethylhydroxylamide**

A solution of Boc-L-(1-naphthyl)-alanine-L-leucine N,O-dimethylhydroxylamide (459 mg), trifluoroacetic acid (5 mL), and thioanisole (2 mL) was stirred at 0°C for 2.5 hours. The solvent was evaporated and the residue was dried under vacuum. A portion of this product (182 mg) was transferred to another flask and was combined with Z-L-(2-naphthyl)-alanine (171 mg), 1-hydroxybenzotriazole monohydrate (99 mg), N-methylmorpholine (0.08 mL), and DMF (10 mL). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (112 mg) was then added at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature for 41 hours. The reaction was quenched with water (20 mL) and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with aqueous 10% HCl, saturated $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$, filtered, and evaporated to afford the product as a white solid. This was then purified by silica gel chromatography (hexane/acetone 80:20, 70:30) to give the title compound (321 mg).

d) **Z-L-(2-naphthyl)-Alanine-L-(1-naphthyl)-Alanine-L-Leucinal**

Z-L-(2-naphthyl)-alanine-L-(1-naphthyl)-alanine-L-leucine-N,O-dimethylhydroxylamide (321 mg) was dissolved in 15 mL dry tetrahydrofuran (THF) and cooled to 0°C. Lithium aluminum hydride (1 M, solution in THF, 1.7 mL) was added and the mixture was stirred at 0°C for 30 minutes. Potassium bisulfate (0.59 g) in 30 mL water was added and the mixture was extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with aqueous 5% HCl, saturated $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$, filtered, and evaporated to afford the product (274 mg) as a white solid.

Example 6

Immunodepletion of the proteasome inhibits the processing of NF-κB,

Monoclonal antibodies against specific components of the proteasome (MCP20, 29K) and a control McAb against hemagglutinin (HA 12CA5) were incubated with Pr(II) extract reconstituted with proteasome activity from the pellet. The immune complexes were removed and the depleted extracts were used in p60 processing reactions as described in Example 4. The results are shown in Figure 4.

Example 7

Purified proteasomes stimulate the processing of p60Tth

Increasing amounts of purified 20/26S proteasomes or a proteasome-enriched fraction from reticulocyte lysate, fraction II, were added alone or were combined with Pr(II) extract in a processing reaction (see Example 4). In addition, processing was inhibited by ATPγS, a non-hydrolyzable analogue of ATP that allows ubiquitination but inhibits proteasome function (lanes 3-5). See Figure 5.

Example 8

The p60Tth precursor protein is ubiquitinated.

In this Example, and in those described above, there are ladder-like bands that appear when the substrate is incubated with extracts lacking proteasome activity (Pr). The ubiquitination of p60 is more pronounced when 7.5 μg of purified ubiquitin (ub) is added to the Pr(II) extract in a processing reaction (lane 5). See Figure 6.

hour prior to the addition of ^{35}S-methionine/cysteine. A typical 20 minute pulse-2 hour chase experiment, immunoprecipitated with anti-p50 Ab followed by SDS-PAGE, is shown in Figure 9. Only proteasome-specific inhibitors block p105 processing; non-specific protease inhibitors do not have an effect. These results were verified *in vitro*.

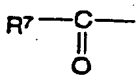
Example 12

Specific inhibitors of the proteasome block activation of NF-κB

HeLa or MG63 cells were pretreated with inhibitors (50 μM) for one hour. Cells were then treated with TNF-α (1000 U/ml) or IFN-γ (1000U/ml) for 30 and 60 minutes, respectively. Whole-cell extracts were prepared and analyzed by an electrophoretic mobility shift assay. The NF-κB site from the interferon-β gene was used to examine NF-κB binding activity and the pIRE site from the IRF-1 gene was used to measure gamma-activated factor (GAF) activity. The inhibitors only block NF-κB activation and have no effect on GAF induction. The results are shown in Figure 10.

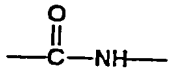
Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention.

3. The method of claim 2 wherein P is

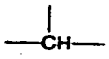


and R^1 is alkyl, aryl, alkaryl, aralkyl, alkoxy, aryloxy, alkaryloxy, or aralkoxy.

4. The method of claim 3 wherein X^1, X^2, and X^3 are



5. The method of claim 4 wherein A is 0 and B^1, B^2, and B^3 are



6. The method of claim 5 wherein R^1 and R^2 are independently selected from the group consisting of alkyl and $-CH_2-R^5$, where R^5 is cyclohexyl or naphthyl.

7. The method of claim 6 wherein R^1 and R^2 are isobutyl.

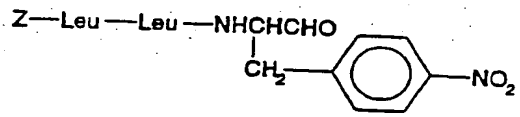
8. The method of claim 2 wherein the proteasome inhibitor is selected from the group consisting of

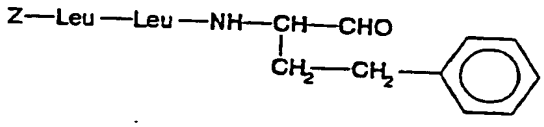
Ac—Leu—Leu—Nle—H

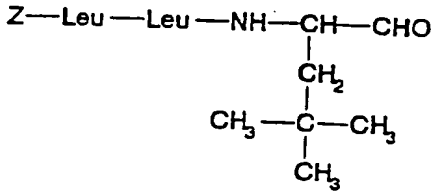
Ac—Leu—Leu—Met—H

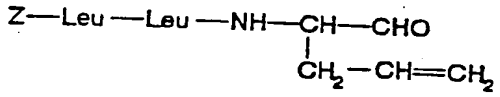
Z—Leu—Leu—Val—H

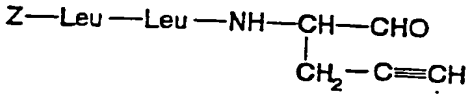
Z—Leu—Leu—Nle—H











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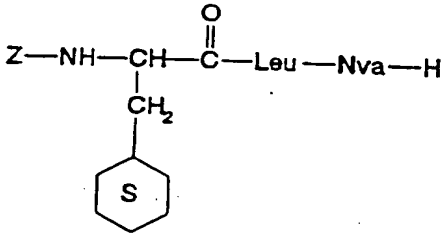

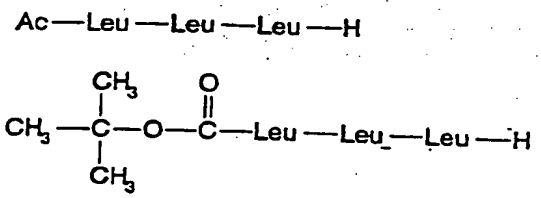

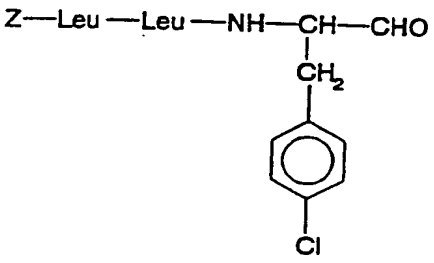

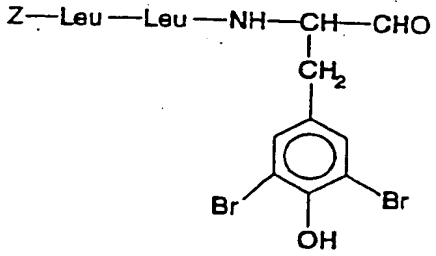

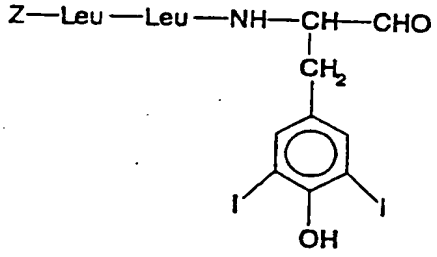

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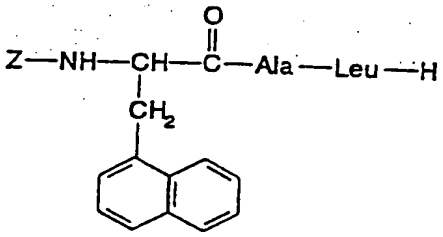

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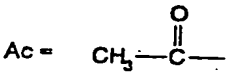
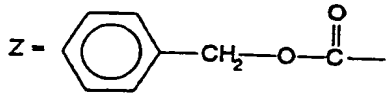


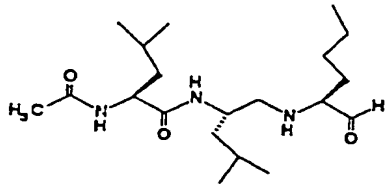


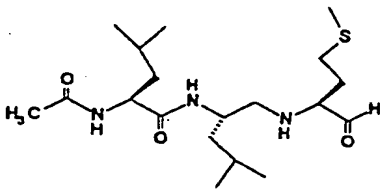
Nle = Norleucine

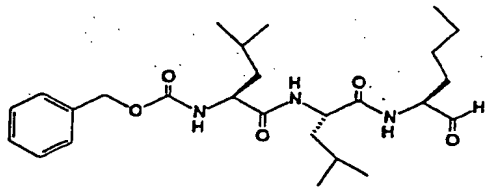
Nva = Norvaline

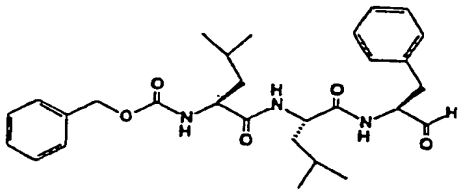
Nal = Naphthylalanine

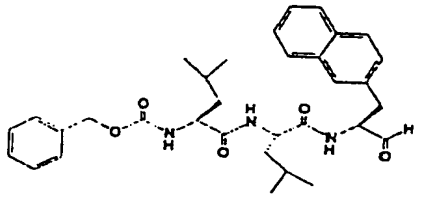
9. The method of claim 2 wherein the proteasome inhibitor is selected from the group consisting of

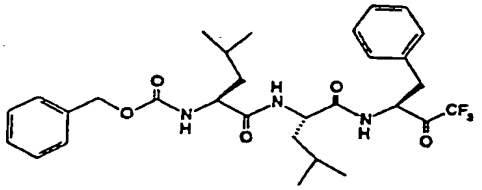


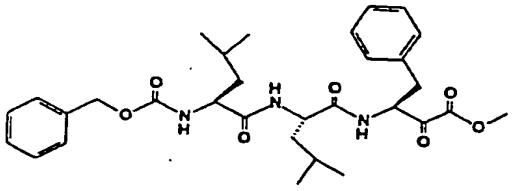












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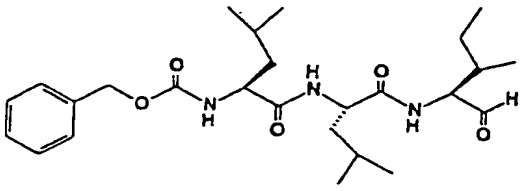

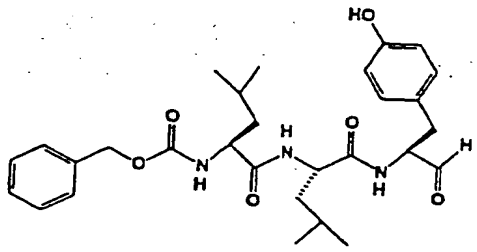

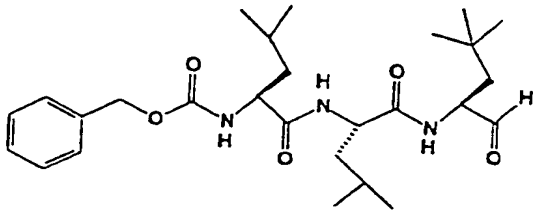

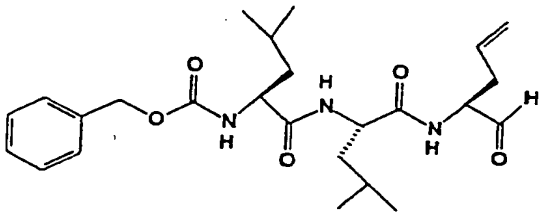

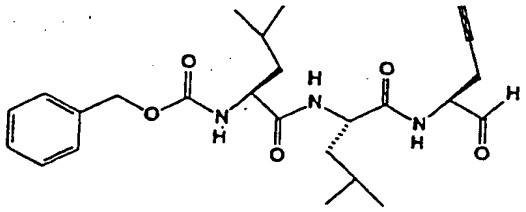

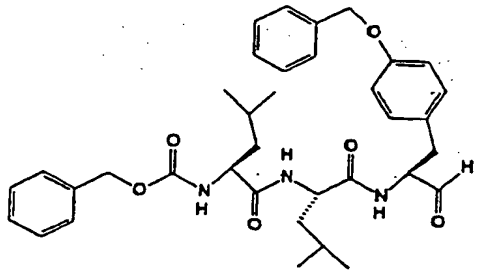

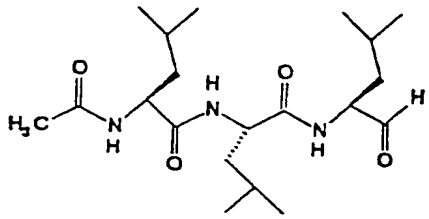

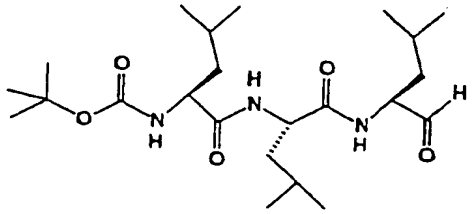

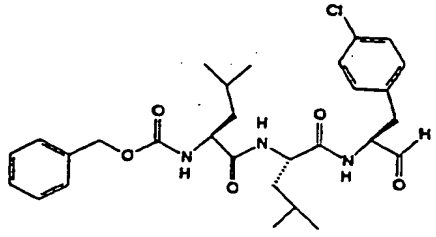

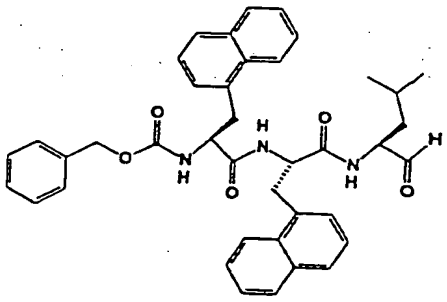

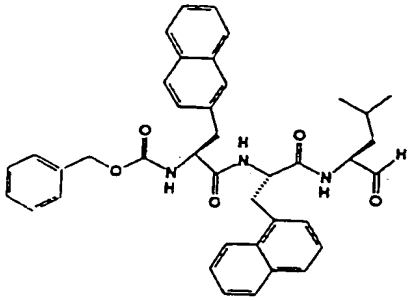

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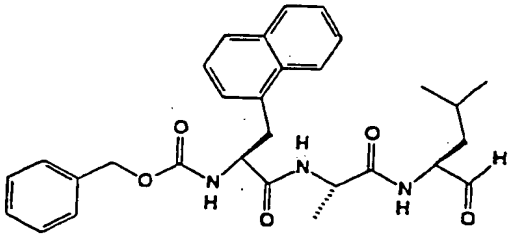

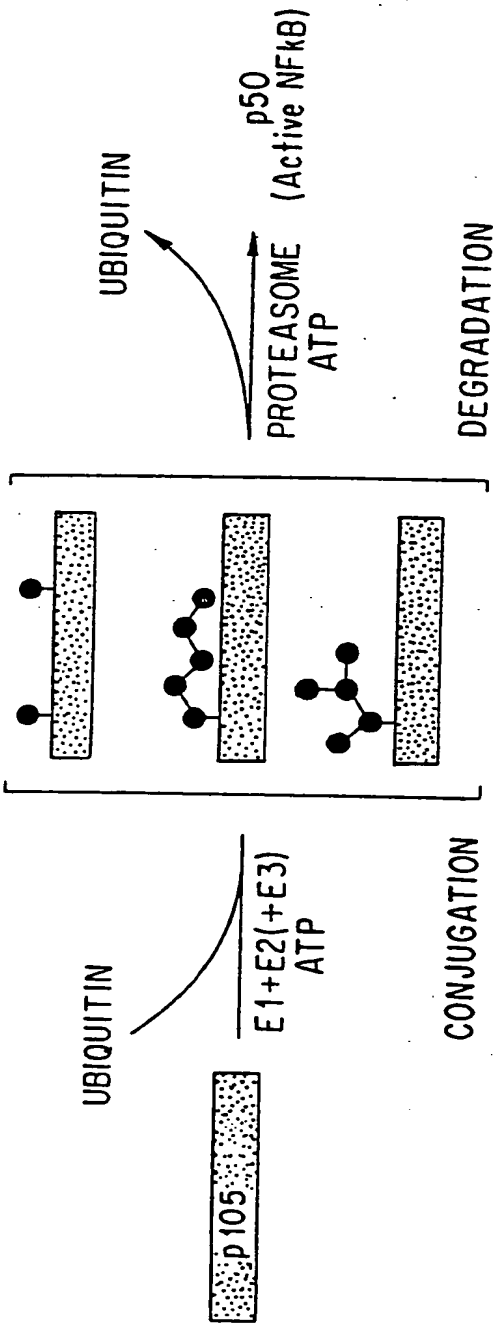
UBIQUITIN
p50
(Active NFkB)
PROTEASOME
ATP
DEGRADATION
UBIQUITIN
E1+E2(+E3)
ATP
CONJUGATION
p105

FIG. 2

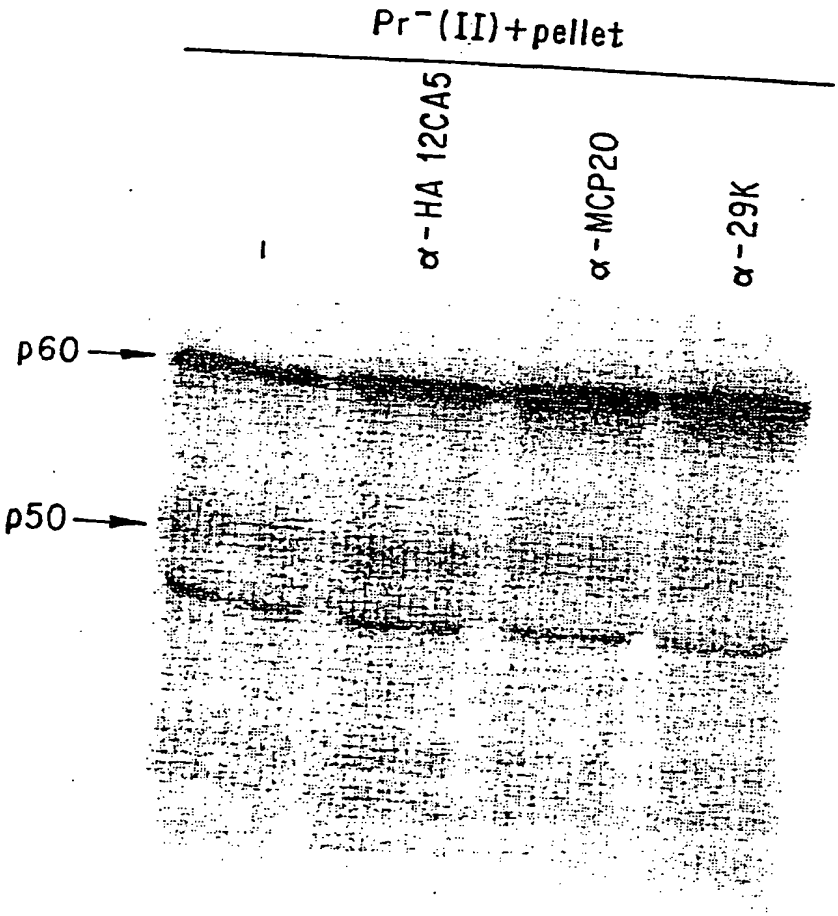

FIG. 4

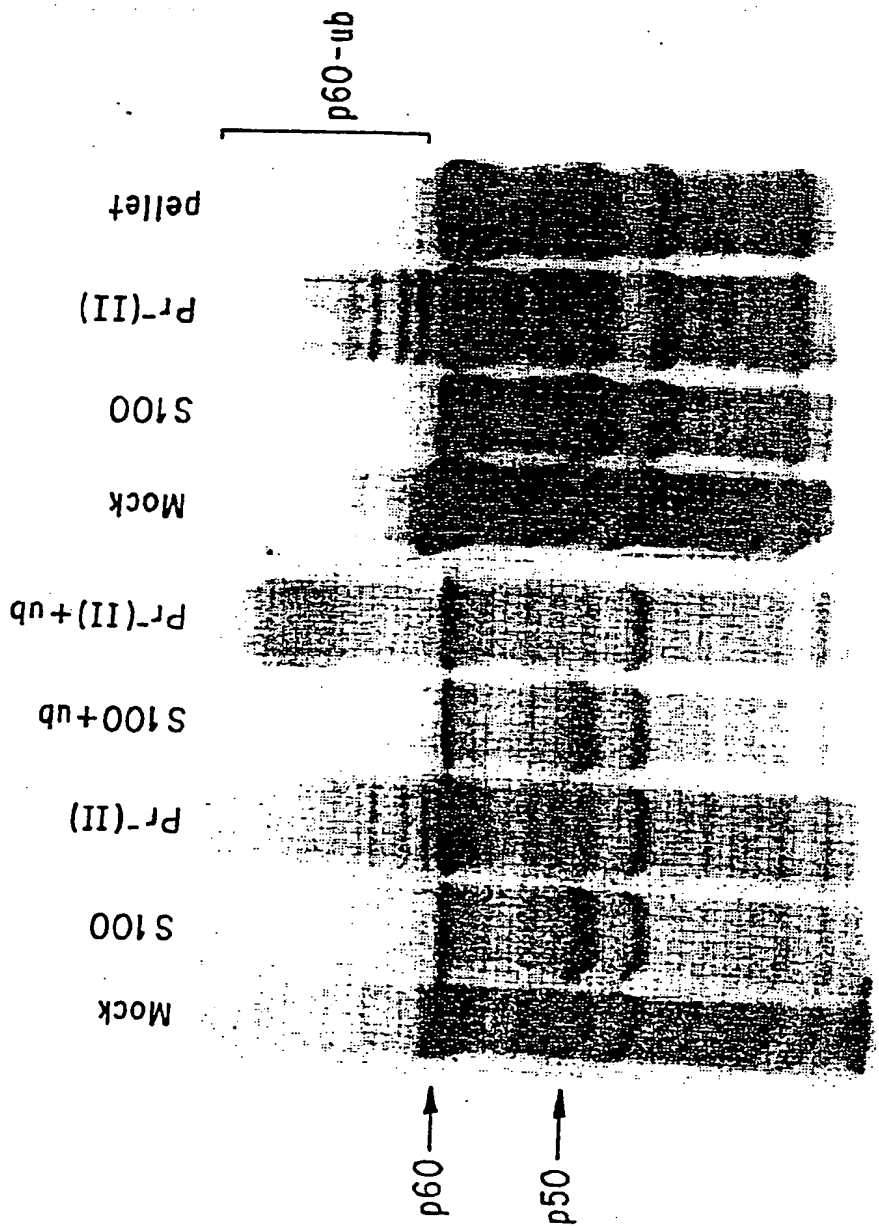

FIG. 6

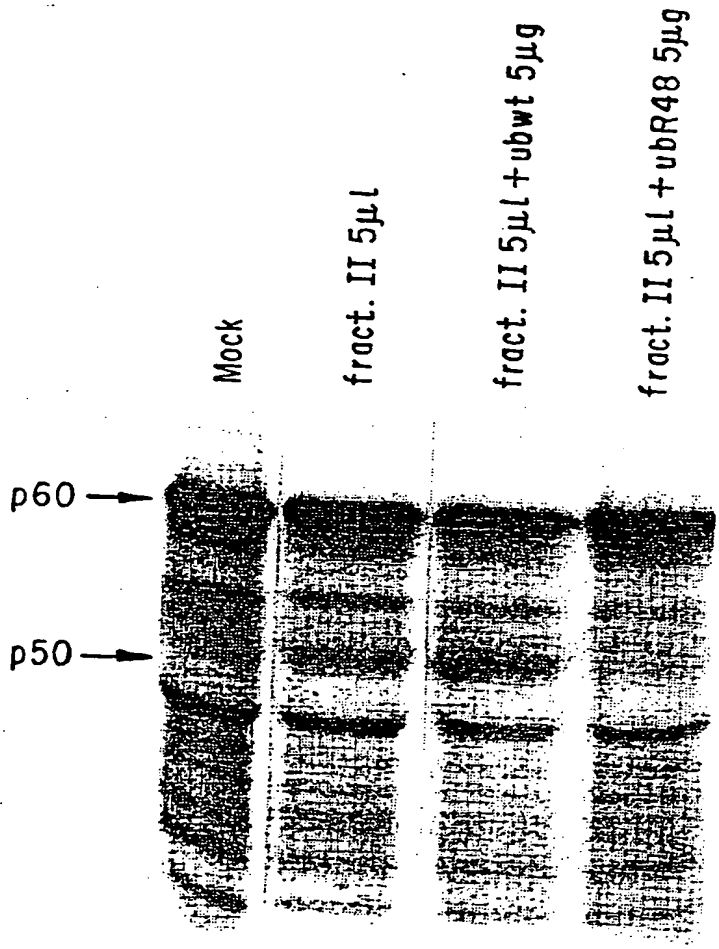

FIG. 7C

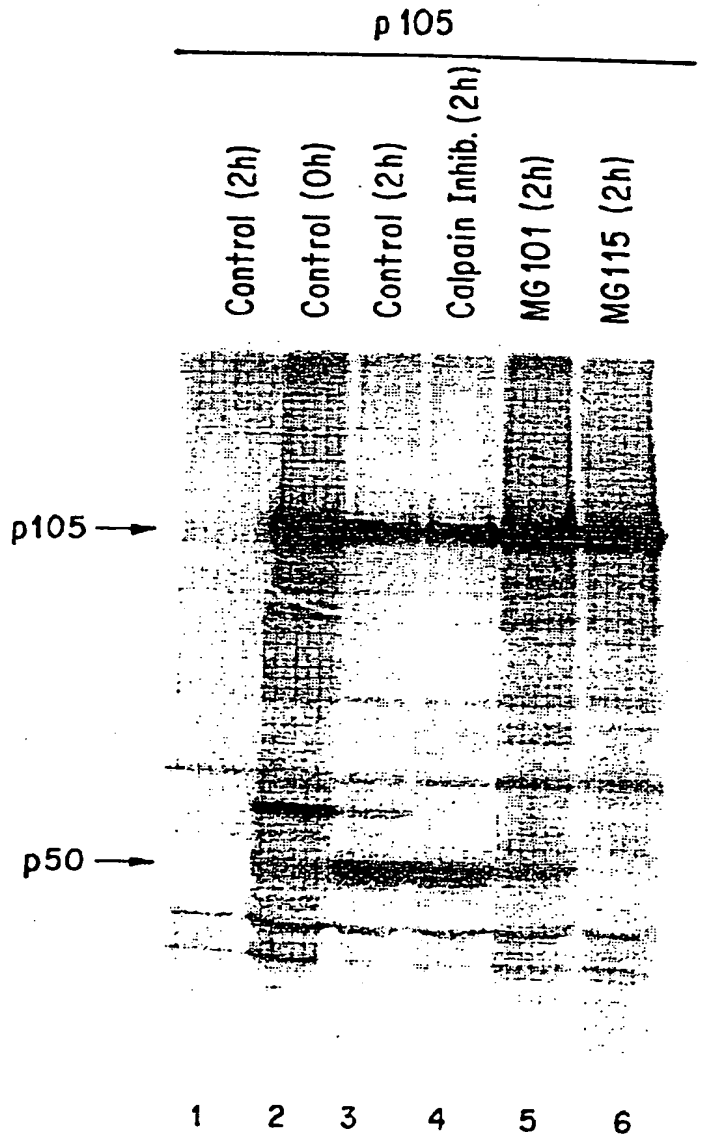
p 105
Control (2h)
Control (0h)
Control (2h)
Calpain Inhib. (2h)
MG101 (2h)
MG115 (2h)
p105
p50
1
2
3
4
5
6
COS cells

FIG. 9

INTERNATIONAL SEARCH REPORT

Int...national application No.
PCT/US95/03315

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/06, 38/07
US CL :514/18
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS
search terms: Pallombella Vito J; structure search of Leu-Leu-Xaa

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Experimental Medicine, Volume 175, Number 5, issued 1992, Schreck et al., "Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells", pages 1181-1194, see entire article.	1-9
Y	Biochemistry, Volume 31, Number 39, issued 1992, Vinitsky et al., "Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex", pages 9421-9428, see entire article.	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 JUNE 1995	29 JUN 1995
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